

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : <b>C12N 15/37, 15/62, A61K 39/23, C07K 13/00</b>		A1	(11) International Publication Number: <b>WO 94/23037</b> (43) International Publication Date: <b>13 October 1994 (13.10.94)</b>
(21) International Application Number: <b>PCT/GB94/00683</b>		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, TJ, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: <b>31 March 1994 (31.03.94)</b>			
(30) Priority Data: <b>9306731.2 31 March 1993 (31.03.93) GB</b>			
(71) Applicant (for all designated States except US): <b>CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; Cambridge House, 6-10 Cambridge Terrace, Regents Park, London NW1 4JL (GB).</b>		Published <i>With international search report.</i>	
(72) Inventors; and			
(75) Inventors/Applicants (for US only): <b>CAMPO, Maria, Severia [IT/GB]; 33 Cleveden Road, Glasgow G12 0PH (GB). JARRETT, William, Fleming, Hoggan [GB/GB]; 60 Netherblane, Blanefield, Glasgow G63 9JP (GB).</b>			
(74) Agent: <b>O'BRIEN, Caroline; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</b>			

(54) Title: **PHARMACEUTICALS BASED ON PAPILLOMAVIRUSES**

(57) Abstract

The invention provides pharmaceutical formulations for the prophylaxis of papillomavirus tumours or lesions. Formulations may be selected from the group consisting of (i) a formulation which comprises a papillomavirus (PV) L2 protein or prophylactically effective fragment thereof and an aluminium compound; (ii) a formulation which comprises a bovine papillomavirus (BPV) L2 protein or prophylactically effective fragment thereof and an aluminium compound; (iii) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an aluminium compound; (iv) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an adjuvant; (v) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an adjuvant; and (vi) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an aluminium compound.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

PHARMACEUTICALS BASED ON PAPILLOMAVIRUSES

The present invention relates to pharmaceuticals based on papillomaviruses.

5 Papillomaviruses induce a variety of lesions both in humans and in animals. Some papillomas, albeit benign, are themselves a clinical problem, such as laryngeal papillomas of children or penile papillomas of bulls and others are known to be a risk factor in the pathogenesis of cancer, as  
10 in the case of flat lesions of the cervix or penile condylomata in humans. Therefore both in human and veterinary medicine, antiviral pharmaceuticals against papillomaviruses eg for prophylactic use to protect against the establishment of a serious infection would be of major  
15 advantage.

Vaccination studies in humans present several problems. First of all experimentation is ethically unacceptable. Secondly, very limited amounts of virus are available as some lesions, in particular those of the  
20 cervix, do not produce viral progeny, and no in vitro system is yet available which allows vegetative replication of virus.

The production of viral proteins in bacteria and the use of synthetic peptides have circumvented this last  
25 problem and have allowed the ongoing analysis of the immune response to papillomavirus infection (see for instance Jenison et al, 1988 J. Virol, 62 p. 2115 and Tindle et al, 1990 J. Gen. Virol, 71 p. 1347.

Effective prophylactic vaccines, both natural (Jarrett

et al, 1990 The Vet. Record, 126 p. 449) and genetically engineered (Pilachinski et al, 1986 Ciba Foundation Symposium Vol. 120 p. 136) have already been produced against bovine papillomaviruses, and regression of Shope papillomas has been achieved by vaccinating rabbits with tumour tissue extracts (Evans et al, 1962 J. Nat. Cancer Inst. 29 p. 277). The bovine system is an excellent model for papillomavirus induced diseases in the human and for medicaments against such diseases in humans given the

5 10 several similarities between the bovine and human disease.

Namely multiple virus types with high lesion specificity (Jarrett et al, 1984 Virol. 136 p. 255), homology of genetic structure, and progression of some lesions to malignancy. The bovine system also presents several  
15 advantages in that cofactors in oncogenesis are known (Campo and Jarrett, 1986 In Papillomaviruses, Ciba Foundation Symposium 120, John Wiley and Sons p. 117) and, above all, direct experimentation is possible (Jarrett, 1985 In advances in Viral Oncology (Ed. G. Klein) 5 p.83).

20 Vaccination has been traditionally regarded as a prophylactic measure. Hosts are immunised against a pathogen and on subsequent exposure to the same organism, the infection is aborted at a preclinical stage. This is usually accomplished in virus infections by a neutralizing  
25 event directed at a superficial epitope on a structural protein. Virus-induced tumours such as papillomas often persist for long periods and are then, in a proportion of cases, rejected. The host may then be immune to

reinfection (Jarrett, 1985 *supra*). The nature of the rejection mechanism and its mediators is unknown, but it is advantageous to induce the rejection mechanism early in a tumour life cycle. In veterinary practice, it has been known for a long time that crude extracts of papillomas could sometimes, but by no means always, cause the rejection of homologous papillomas (Olson et al, 1959 *Am. J. Vet. Res.* 21 p. 233, Evans et al, 1962 *supra*). These experiments were carried out before it was known that there were different types of papillomavirus in a species (Jarrett et al, 1984 *supra*; de Villier, 1989 *J. Virol.* 63 p. 4898), and that these are probably all immunologically type-specific (Jarrett et al. 1990 *The Vet. Record* 126 p. 473; Jenison et al, 1988 *supra*).

Papillomaviruses can infect and cause tumours in both cutaneous and mucous epithelia. In man and animals mucosal papillomas are often serious lesions which tend to run a prolonged course, as in the case of human laryngeal, genital and cervical papillomas (Steinberg, 1987 *In the Papovaviridae Vol. 2* (ed. N.P. Salzman and P.M. Howley) p. 265; zur Hausen, 1991 *Virol.* 184 p. 9). These may be causal factors in the subsequent development of malignancies, as in the case of human papillomavirus (HPV) type 16-associated cervical carcinoma in women (zur Hausen, 1991 *supra*), and bovine papillomavirus (BPV) type 4-associated alimentary cancer in cattle (Campo and Jarrett, 1986 *supra*). Clearly, there is an urgent need for pharmaceuticals against papillomavirus diseases.

Despite recent limited success (Kreider et al, 1986 J. Virol. 59 p. 369; Sterling et al, 1990 J. Virol. 64 p. 6305; Meyers et al, 1992 Science 257 p. 971; Dollard et al, 1992 Genes and Development 6 p. 1131), papillomaviruses are 5 notoriously refractory to growth in cultured cells (Teichaman and LaPorta, 1987 In The Papovaviridae, Vol. 2 (ed. N.P. Salzman and P.M. Howley) p. 109) and the consequent lack of viral reagents has delayed the analysis of the immune response to the infection. The recent 10 availability of recombinant technology has allowed the production of both early and late viral proteins in large amounts and in a purified form (Tindle et al, 1990 supra.; Jarrett et al, 1991 Virol. 184 p. 33; Ghim et al, 1992 Virology 190 p. 548; Stacey et al, 1992 J. Gen. Virol. 73 15 p. 2337) and thus has for the first time made it possible to study their immunising potential. It has already been shown that in cattle it is possible to use viruses and tumour extracts to induce both protection against, papillomavirus-induced cutaneous lesions (Jarrett et al, 20 The Vet Record 1990 126 p. 473; The Vet Record 1990 126 p. 449) and to use recombinant proteins to induce both protection against and rejection of PV-induced cutaneous 25 lesions (Virology 1991, 184 p. 33). The applicants now show that one can vaccinate in a practically significant fashion against papillomavirus for example the mucosal virus BPV-4, which in the field is a causative factor of squamous cell carcinoma of the upper alimentary canal (Campo and Jarrett, 1986 supra).

The present invention provides a pharmaceutical formulation for the prophylaxis of papillomavirus tumours or lesions selected from the group consisting of: (i) a formulation which comprises a papillomavirus (PV) L2 protein or prophylactically effective fragment thereof and an aluminium compound, (ii) a formulation which comprises a bovine papillomavirus (BPV) L2 protein or prophylactically effective fragment thereof and an aluminium compound; (iii) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an aluminium compound; (iv) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an adjuvant; (v) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an adjuvant; (vi) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an aluminium compound; (vii) a formulation which comprises a PV L2 protein or prophylactically effective fragment thereof and an adjuvant; (viii) a formulation which comprises an HPV L2 protein or a prophylactically effective fragment thereof and an adjuvant; (ix) a formulation which comprises an HPV L2 protein or a prophylactically effective fragment thereof and an aluminium compound; (x) a formulation which comprises an HPV-16 L2 protein or a prophylactically effective fragment thereof and an adjuvant; (xi) a formulation which comprises an HPV-16 L2 protein or a prophylactically effective fragment thereof and an

aluminium compound.

The pharmaceutical formulation may comprise a PV L2 protein or a prophylactically effective fragment thereof and an aluminium compound. The PV may be BPV. The BPV may  
5 be BPV-4.

The papillomavirus protein or prophylactically effective fragment thereof may be in the form of a fusion protein with a different co-protein. The co-protein may be glutathione-S-transferase.

10 The papillomavirus protein or prophylactically effective fragment thereof may be produced by recombinant DNA techniques.

The aluminium compound may comprise a mixture of aluminium hydroxide and aluminium phosphate. There may be  
15 3% aluminium hydroxide and 2% aluminium phosphate. The aluminium compound may be aluminium gel.

Also provided is the use of a pharmaceutical formulation as above in medicine for the prophylaxis of papillomavirus tumours or lesions

20 Also provided is use of (i) a PV L2 protein or prophylactically effective fragment thereof and an aluminium compound; or (ii) a BPV L2 protein or prophylactically effective fragment thereof and an aluminium compound; or (iii) a BPV-4 L2 protein or prophylactically effective fragment thereof and an aluminium compound; or (iv) a BPV-4 protein or prophylactically effective fragment thereof and an adjuvant; or (v) a BPV-4 L2 protein or prophylactically

effective fragment thereof and an adjuvant; or (vi) a BPV-4 protein or prophylactically effective fragment thereof and an aluminium compound; or (vii) a PV L2 protein or prophylactically effective fragment thereof and an  
5 adjuvant; or (viii) an HPV L2 protein or a prophylactically effective fragment thereof and an adjuvant; or (ix) an HPV L2 protein or a prophylactically effective fragment thereof and an aluminium compound; or (x) an HPV-16 L2 protein or a prophylactically effective fragment thereof and all  
10 adjuvant; or (xi) an HPV-16 L2 protein or a prophylactically effective fragment thereof and an aluminium compound in the production of a vaccine for the prophylaxis of papillomavirus tumours or lesions.

Also provided is a method of immunising a mammal  
15 against papillomavirus tumours or lesions which comprises the administration of a pharmaceutical formulation as above in a therapeutically effective dosage.

Vaccines based upon the pharmaceutical formulations  
for prophylactic use as described above, can be prepared in  
20 accordance with methods well known in the art. Likewise, dosage rates can be determined according to known methods. Attention is directed to New Trends and Developments in Vaccines, Editors A. Voller and H. Friedman, University Park Press, Baltimore, 1978 and to Remington's  
25 Pharmaceutical Science by E.W. Martin.

In order that the invention is more clearly understood, it will be further described by way of example only, and not by way of limitation, with reference to the

following figures.

Figure 1 shows results for vaccination with  $\beta$ -gal-early (E1, E2, E4 and E7) fusion proteins. A, animal No. 132 from group 3 (control. B, animal No. 146 from group 1 (pre-challenge vaccination). C, animal No. 139 from group 2 (post-challenge vaccination). Bar legend is in Figure. D, pURE7. The recombinant plasmids containing E1, E2 and E4 ORFs are not shown.

Figure 2 shows results for vaccination with  $\beta$ -gal-E7. A, mean number of all tumours in vaccinated and control animals. B, mean number of stage 1 tumours. C, mean number of stage 2 tumours. D, mean number of stage 3 tumours. Squares, vaccinated animals; diamonds, control animals. In a repeated measurement analysis, P=0.02 in C and 0.04 in D.

Figure 3 shows results for vaccination with E7+L2 and L2. A, pGEX recombinant plasmids. B-D, number of tumours in calves eleven weeks after challenge; B, group 1 (E7+L2 vaccine); C, group 2 (L2 vaccine); D, group 3 (control).

#### 20 Materials and Methods

All genetic manipulation procedures are carried out according to standard methods described in "Molecular Cloning", A Laboratory Manual eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989

25 In the following it is described how the applicants produced recombinant BPV-4 peptides in Escherichia coli using pAT153 as a source of E7 and L2. Alternatively the invention may be put into effect by use of the L2 and E7

sequence information available from EMBL sequence database (accession no. X59063).

#### Calves

Calves of mixed breed, approximately 12 weeks old, 5 were obtained from papilloma-free sources. They were randomly assigned to groups and housed in separate, clean, well-ventilated pens in an isolation unit. All the calves were bled on arrival and at three to four weeks intervals thereafter for haematological analysis. The animals were 10 cared for in complete accordance with the directives of the Home Office of Great Britain.

#### Production of recombinant BPV-4 peptides in Escherichia coli

##### (i) β-Galactosidase E7 Fusion Proteins

15 The E1, E2, E4 and E7 open reading frames (ORFs) of BPV-4 were cloned in the pUR plasmid series (Ruther and Muller-Hill, 1983 EMBO J. 2 p. 1791). The cloning of the E1, E2 and E4 ORFs is not described.

The ORF encoding the E7 peptide was isolated by 20 digesting the BPV-4 genome cloned in pAT153 (Campo M.S. et al, 1982 J. Gen. Virol. 63, p. 255) with BamHI. The ORF encoding the E7 peptide was isolated as a BsrI fragment encompassing nucleotides (nts) 652-1249 and cloned in pUR 278 (Ruther and Mullter-Hill 1983 supra) via addition of 25 BamHI linkers. The recombinant plasmid was transfected into Escherichia coli JM109 (Promega Ltd, Southampton, UK). The bacteria were grown to mid-log phase in L-broth supplemented with 100 µg/ml ampicillin and induced to

10

express the  $\beta$ -gal-fusion protein by addition of 100  $\mu$ g/ml IPTG for 1-4hr.  $\beta$ -gal-E7 was prepared by suspending bacteria in lysozyme buffer (25% sucrose, 10mM MgCl<sub>2</sub>, 50 mM Tris HCl pH 8.0, 1mg/ml lysozyme) containing 300  $\mu$ g/ml DNase I. The fusion peptide was pelleted following cell lysis in 0.25% NP40, 0.125% deoxycholate, 0.25M NaCl, Tris HCl pH 7.2 and subsequent washes in 1.75M Guanidine HCl, 1M NaCl, 1% Triton X100.

5 (ii) Glutathione-S-transferase (GST) Fusion Proteins

10 The E7 and L2 ORFs were cloned in pGEX (Smith and Johnson, 1988 Gene, 67 p.31).

15 The ORF encoding the E7 peptide was isolated as described above and cloned in pGEX 3X (Pharmacia Ltd, Milton Keynes MK9 3HP, UK) by blunt-end ligation (see Fig 3).

20 The ORF encoding the L2 peptide isolated by digesting the BPV-4 genome cloned in pAT 153 (Campo et al 1982 supra) was obtained as a BamHI-EcoRI fragment encompassing nt 3987-5585 and cloned in its entirety in pGEX 2T (Pharmacia Ltd, Milton Keynes MK9 3HP, UK), and also as three Dde I fragments: (i) a 5' end fragment (nt 4042-4610) in pGEX 2T; (ii) a middle fragment (nt 4610-4989) in pGEX 3X; and (iii) a 3' end fragment (nt 4989-5629) in pGEX 3X (see Fig. 3).

25 The GST fusion peptides represent the E7 protein from amino acids -21 to 98 (GST-E7); the L2 protein from amino acids -8 to 542 (GST-L2w) amino acids 11-200 (GST-L2a); amino acids 201-326 (GST-L2b); and amino acids 327-542 (GST-L2c). Transformation and growth of bacteria and production of

GST-fusion peptides were carried out as outlined for  $\beta$ -gal E7. The peptides were prepared in bulk by pelleting inclusion bodies from bacteria suspended in lysozyme buffer containing DNase I at 300  $\mu$ g/ml and deoxycholate at 1mg/ml, followed by washing in 0.5% Triton X100/10mM EDTA, pH 8.0. In all cases fusion peptides were suspended by boiling and sonication in 5% SDS, 5mM B-Mercaptoethanol, 50mM Tris HCl, pH 8.0 prior to vaccination. Yields were 2-3mg per gram wet weight of bacteria and 50-70% purity was routinely achieved.

#### Characterization of fusion proteins

The  $\beta$ -gal-E7, GST-E7 and GST-L2 peptides were characterised by SDS-PAGE electrophoresis. The molecular weights of the fusion peptides were all in agreement with prediction.  $\beta$ -gal-E7 and GST-E7 were characterised immunologically using rabbit, bovine or murine antisera raised against  $\beta$ -gal-E7 or GST-E7 or GST-E7, in ELISA or Western blot analysis following absorption with the appropriate bacterial protein. The immune sera reacted positively with both the homologous and the heterologous antigen, showing that the fusion proteins were immunologically active and the sera were specific for E7 (see Table 1). GST-L2 antigens were reactive in ELISA tests with homologous bovine sera, as was a  $\beta$ -gal L2 fusion protein, showing that GST-L2 was active immunologically and the sera were specific for L2 (see Table 1).

Table 1  
FUSION PROTEINS ARE IMMUNOLOGICALLY ACTIVE

		<u>blank</u>	<u>no Ag</u>	<u>no Ab</u>	<u><math>\beta</math>-gal</u>	<u><math>\beta</math>-gal:E7</u>	<u>GST</u>	<u>GST-E7</u>
Animal 5 ( $\beta$ -gal-E7)	pre-Immune	0.064	0.104	0.105	0.153	0.206	0.132	0.134
	Immune	0.058	0.192	0.174	0.718	2.892	0.112	0.886
Animal 16 (GST-L2)	pre-Immune	0.064	0.149	0.124	0.15	0.231	0.284	0.317
	Immune	0.087	0.157	0.105	0.12	1.512	0.724	2.694

Microtitre wells were coated with 500  $\mu$ g/well of E7 fusion protein.  $\beta$ -gal and GST were used in equivalent amounts. The wells were incubated overnight with the appropriate bovine sera and anti bovine IgG conjugated with Alkaline Phosphatase was added for 1 hour. Animal 5 is from exp 3, group 1; animal 16 is from exp 4, group 2.

#### Experimental design

##### (i) Vaccination with E1, E2, E4, E7 early fusion proteins

Eighteen animals were divided into three groups of six animals each. Each animal in group 1 was inoculated in the posterior left flank with 1 ml of PBS containing 1 mg of a cocktail of  $\beta$ -gal-early (E1, E2, E4 and E7) fusion proteins emulsified in 1 ml of Freund's incomplete adjuvant (FIA); vaccination (boost) was repeated with a fortnight in the posterior right flank. Two weeks after the boost, all of the animals in each group were challenged in the palate (Jarrett et al, 1990 The Vet Record, 126 p. 473) with  $10^{12}$  particles of BPV-4. Animals in group 2 were vaccinated as above two weeks after challenge, and boosted after a further two weeks. BPV-4 was purified from oesophageal papillomas and typed as described previously (Campo et al, 1980 Nature, 286, p. 180) and the concentration of viral particles was estimated by the electron microscope assay (Jarrett et al, 1990 The Vet Record, 126 p. 449). Each animal was examined every three to four weeks, the papillomas were counted and their size measured as previously described (Jarrett, 1985 supra).

##### (ii) Vaccination with $\beta$ -gal-E7

Nineteen animals were divided into two groups of eleven and eight animals each. The eleven calves in group 1 were each given a 1mg/1ml suspension of the  $\beta$ -gal-E7 fusion protein emulsified in 1ml of FIA into the right quadriceps muscle. The vaccination was repeated four weeks later into the left quadriceps muscle (boost). Fourteen

days after the boost, all the animals in group 1 and 2 were challenged in the palate with  $10^{11}$  BPV-4 particles at ten sites ( $10^{10}$  particles per site). The animals were examined and the papillomas monitored as above.

5 (iii) Vaccination with GST-E7 and GST-L2

Forty-seven animals were divided into two groups of fifteen animals each (group 1 and 2) and one group of seventeen animals (group 3). The calves of group 1 were each given a 2 ml suspension containing 1 mg of the GST-E7 fusion protein and 1 mg in total of the GST-L2 fusion peptides (the ratio of the GST-fusion peptides L2w:L2a:L2b:L2c was approximately 1:5:5:5) plus 2 ml of FIA into the right quadriceps muscle. The vaccination was repeated four weeks later into the left quadriceps muscle.

10 The calves of group 2 were each given 1 mg of the GST-L2 fusion proteins as described above. Two weeks after the boost the animals in all three groups were inoculated in the palate at ten different places with a total of  $10^{11}$  BPV-4 particles ( $10^{10}$  particles per site). The animals were

15 examined and the papillomas monitored as above.

20 (iv) Vaccination with GST-L2 in aluminium gel

Thirty-six animals were divided into three groups of twelve animals each. The calves of group 1 were each given a 3 ml suspension containing 1 ml of GST-L2 fusion peptides at 1 mg/ml in the same ratio as above, 1 ml of 40 mM TRIS-HCl- 0.33% NaCl, and 1 ml of aluminium gel, made up of equal volumes of 3% aluminium hydroxide and 2% aluminium phosphate (Intervet UK Ltd, Cambridge Science Park,

Cambridge CB4, UK). The vaccination was repeated four weeks later. The calves of group 2 were vaccinated as above, but with only 100 µg of L2 fusion peptides per animal. The calves of group 3 were the control. The challenge was as above.

5

### Results

Immunization with the E7 protein inhibits papilloma development and causes papilloma rejection

E7 is the major transforming protein of BPV-4 in vitro (Jagger et al, J. Gen. Virol. 71 p. 3041) and is expressed throughout the different development stages of alimentary canal papillomas, pointing to its importance for the maintenance of the proliferative state also in vivo. It is homologous to the oncoprotein E7 of human papillomavirus type 16 (HPV-16) (Jagger et al, 1990 supra; Jackson et al, 1991 Molecular Carcinogenesis 4 pg. 382), the virus most often associated with squamous cell carcinoma of the uterine cervix in women (zur Hausen, 1991 supra). Because of its pivotal role in cell transformation, E7 may be a target for cell-mediated immune responses leading to tumour rejection, both in BPV-4 and in HPV-16.

15

The applicants conducted two pilot experiments, which will be described briefly, and a third experiment with larger numbers of animals, which will be described in more depth.

In the first experiment, the applicants vaccinated calves with a cocktail of BPV-4 early proteins E1, E2, E4

and E7, synthesized in bacteria as  $\beta$ -galactosidase fusion products (Ruther and Muller-Hill 1983 supra; Jarrett et al, 1991 Virol., 184 p. 33). Six calves in group 1 were vaccinated before being challenged in the mouth with BPV-4, and six calves in group 2 were vaccinated after challenge. Six control calves in group 3 were not vaccinated and were challenged with BPV-4 (see Table 2). Four to six weeks after challenge, all the animals developed plaque-like lesions, which are stage 1a of alimentary canal papilloma development (Jarrett, 1985 supra). The lesions were confined to the injection sites. During the next twenty weeks, the lesions of the control animals grew in number and size, with secondary spread in the palate; and went through the well recognised stages of papilloma growth: raised plaques (stage 1b), papillomas up to 2mm in length (stage 2) and papillomas bigger than 2mm (stage 3). Stage 2 and stage 3 papillomas are the mature virus-producing tumours.

20

Table 2

VACCINATION STRATEGY

<u>Group</u>	<u>Exp 1</u>	<u>Exp 2</u>	<u>Exp 3</u>	<u>Exp 4</u>	<u>Exp 5</u>
1	(PR)E1-2-4-7(6)	E7(6)	E7(11)	E7+L2(15)	L2*(12)
2	(PS)E1-2-4-7(6)	E2(6)	nv(8)	L2(15)	L2**(12)
25	3	nv(6)	nv	nv(17)	nv(12)

The number of animals in each group is in parentheses. The animals were inoculated twice with 1 mg of protein, except

in exp 5, group 2 where they received two inoculations of 100 µg each (L2\*\*). The adjuvant was FIA in all cases, except in exp 5 (L2' and L2\*\*) were it was aluminium gel (Intervet UK, Ltd). In exp 1, PR indicates vaccine given pre-challenge and PS indicates vaccine given post-challenge; in exp 2-5, the vaccine was always administered pre-challenge nv. no vaccine.

Details of the papilloma developmental stages and their significance in the tumour life cycle have been described (Jarrett, 1985 supra). Approximately thirty weeks after challenge, natural involution of papillomas started to take place with decrease in size and number of lesions, and the animals were essentially papillomatosis-free by week fifty-four. The development of papillomas in a representative animal from this group is shown in Figure 1A. All the vaccinated animals, whether vaccinated before or after challenge, had approximately the same number of plaques as the control animals, indicating that the virus was equally infectious and the calves equally susceptible to infection in the three groups. However most of the lesions in the vaccinated animals did not develop to full size, particularly in group 1, and were most rejected by 40-47 weeks after challenge, much earlier than in the control animals. The development of lesions in representative animals from the vaccinated groups are shown in figure 1B and C. Two conclusions could be drawn from these results: that therapeutic vaccination was possible.

and that, within the time intervals used in this study, the time of vaccination relative to challenge was not critical.

To ascertain which one of the four early proteins was responsible for tumour regression, the applicants  
5 vaccinated six calves with  $\beta$ -gal-E7 and six with  $\beta$ -gal-E2 before challenge; six animals were the control group (see Table 2). The E2-vaccinated animals did not behave any differently from the control group, while in the E7-vaccinated calves most of the lesions did not grow beyond  
10 stage 1a and were rejected earlier than either in the control or in the E2-vaccinated animals (data not shown).

In the third larger experiment, eleven calves were vaccinated with  $\beta$ -gal-E7 before challenge and eight calves were kept as controls (see Table 2). In this experiment  
15 plaques and raised plaques will be considered together as stage 1 lesions. As previously, all the animals developed the same number of plaques four weeks after challenge, confirming that they were all equally susceptible to infection (see fig. 2A). After ten weeks, the number of  
20 stage 1 lesions fell sharply in both groups, concomitant with the increase in the number of stage 2 lesions (see Figs. 2B and C). In the control group, the stage 2 lesions progressed to stage 3 lesions in the following ten weeks (see Fig. 2D) while the number of stage 1 lesions remained  
25 constant (see Fig 2B). There was no secondary spread, due to the smaller doses of challenge virus (see Materials and Methods ). After five more weeks (twenty-five weeks post challenge), stage 3 and stage 2 papillomas started

regressing (see Figs 2C and D). In the E7-vaccinated group, by week 10 after infection, the number of stage 2 lesions was higher and the number of stage 3 lesions lower than in the control group (see Figs 2C and D). Thirteen weeks post infection, the number of stage 2 papillomas decreased considerably, without concomitant increase in stage 3 papillomas (see Figs 2C and D). In the E7-vaccinated animals therefore, progression from stage 2 to stage 3 lesions was being dramatically reduced and stage 2 papillomas were regressing before reaching full maturity. The difference between the control and the vaccine group was observed not only when the number of stage 2 and stage 3 papillomas per group were considered, as in Fig. 2, but also when the percentage of stage 3 papillomas or the percentage of animals with stage 3 papillomas was considered (see Table 3).

In the three different experiments, a total of twenty-nine animals was vaccinated with E7. Twenty-two (76%) either did not develop mature papillomas or rejected them earlier than the control animals. It may therefore be concluded that vaccination with E7 reduces tumour growth and induces premature tumour regression thus effectively providing a treatment for papillomas.

Vaccine E7 is effectively presented to the immune system

Vaccination with E7 is accompanied by both a humoral and a cellular immune response to the vaccine. Both responses appear much earlier and have a greater amplitude in the vaccinated animals than in the control calves.

Whereas all of the vaccinated animals had serum antibodies to E7, serum antibodies to E7 were not in all of the control animals and some remained negative throughout the course of the experiment. Vaccine E7 is therefore presented to both effector arms of the immune system, while viral E7 is poorly presented. This may explain the efficacy of the therapeutic vaccine. The humoral and cell-mediated immune response to E7 and the mapping of the B- and T-cell epitopes will be presented in greater detail elsewhere.

**Immunisation with the L2 protein prevents papilloma formation**

L2 is the minor capsid protein of papillomavirus. Although the detailed molecular structure of the virion is not yet known, there is circumstantial evidence that a domain(s) of the protein is exposed on the surface of the virus (low titer virus-neutralizing L2 antibodies have been found in rabbits immunised with recombinant L2 protein (Christensen et al, 1991 Virology 181 p. 572; Lin et al, 1992 Virology 187 p. 612)). The applicants have shown that vaccination with the L2 protein of the cutaneous papillomavirus BPV-2 induced early rejection of fibropapillomas of the skin, accompanied by infiltrates of immune cells in the regressing warts (Jarrett et al, 1991 Virology 184 p. 33).

L2 and E7 were produced in bacteria as glutathione-S-transferase (GST) fusion proteins (Smith and Johnson, 1988 supra). Fifteen animals were vaccinated with GST-L2,

fifteen with GST-L2+GST-E7, and seventeen were kept as control (see Table 2). All of the animals were challenged with equal amounts of virus from the same stock. The animals were examined four, seven and eleven weeks after challenge. In group 3, which had received no vaccine, 13 out of 17 calves developed lesions approximately four weeks after virus infection. These developed through the usual stages (see Table 4). Eleven weeks after challenge, when the experiment was terminated, one of the four animals that were free of lesions at four weeks had only one stage 1 lesion and three had no lesions at all (see Table 4 and Fig. 3). Of the fifteen animals in group 1 (L2+E7), two developed stage 1 lesions which subsequently disappeared, one developed stage 2 lesions, which were still present at the end of the experiment, and twelve did not develop any tumours at all (see Table 4 and Fig. 3). In group 2 (L2) by the end of the experiment one calf had five stage 1 and one stage 2 lesions, and the remaining fourteen animals were completely tumour-free (see Table 4 and Fig. 3). The 28 vaccinated animals with no tumours were still papilloma-free forty-four weeks after challenge, whereas the control animals still had papillomas. Thus the L2 and L2+E7 vaccines conferred a high degree of protection, an effect almost certainly due to the L2 protein, as the E7 vaccine has no protective effect by itself (see above). This was confirmed by a second L2 vaccination experiment, in which only the L2 peptides were used, the amount of antigen was decreased to 100 µg per inoculation per animal and the

adjuvant was changed to aluminium gel (see Table 2). Most animals in group 1 (11 out of 12) and all the animals in group 2 (12 out of 12) were completely protected from challenge and tumour-free, whereas 11 out of 12 control 5 animals developed papillomas four weeks after challenge (data not shown). The L2 peptides therefore provide a powerful prophylactic vaccine. The results show that it is possible to reduce the dosage of L2 when administered in aluminium salts, while maintaining a strong prophylactic 10 effect.

Thus the applicants have successfully achieved prophylactic and therapeutic immunisation against mucosal bovine papillomaviruses.

This has been accomplished in an animal host against 15 its own natural pathogen. Cattle and their papillomaviruses have co-evolved and the immunological response observed in experimental conditions mimics the one observed in nature and is therefore biologically significant (Jarrett et al, 1991 Virology, 184 p. 33; 20 Campo, 1991 Cancer Cells, 3, p. 421).

The success obtained against the mucosal virus BPV-4 in the face of heavy challenge is particularly remarkable and of special relevance to the possible use of vaccines against genital papillomavirus in human subjects. There 25 are several similarities between the BPV-4 and the HPV-16 systems. Both viruses infect mucous epithelia, giving rise to lesions that can neoplastically transform. In both viruses the major transforming functions, as defined in in

vitro systems, are encoded by the E7 gene. Furthermore, the two E7 proteins show amino acid homology.

Virtually complete protection against BPV-4 infection was achieved by immunisation with the L2 protein.

5 Vaccination with the L2 protein of the cottontail rabbit papillomavirus (CRPV) has also been shown to have protective effect in the rabbit (Christensen et al, 1991 supra; Lin et al, 1992 supra), although to a more limited extent than in cattle. Taken together, these two sets of  
10 results suggest that L2 of HPV might have a similar effect in humans.

Immunisation with BPV-4 E7 induces rejection of established tumours which is accompanied by a strong cellular immune response. Vaccination of rats and mice  
15 with the HPV-16 E7 gene caused growth retardation and partial regression of tumours induced by HPV-16 transformed cells (Meneguzzi et al, 1991 Virology 191 P. 62-69; Chen et al, 1991 PNAS 88, p.110), and, more recently, vaccination of mice with HPV-16 E7 elicited a delayed type  
20 hypersensitivity response specifically directed against the E7 antigen. Therefore E7 of different papillomaviruses can elicit the appropriate immune response to induce tumour rejection.

Table 3

E7 VACCINE

## WEEKS POST CHALLENGE

	WEEKS POST CHALLENGE						
Lesion Stage	4		7		10		13
	1	2	3	1	2	3	
Mean number of tumours in vaccinated group	6.3 [1.2]	0 0	5.8 [1.3]	3.9 [1.3]	0 0	0.3 [0.3]	8.7 [0.5] 0.3 [0.2]
Mean number of tumours in control group	3.9 [1.3]	1.8 [1.5]	0 0	8 [1.4]	0.1 [0.1]	1.1 [1.1]	2.1 [1.1] 5.4 [1.6] 2.5 [1.5]
Vaccinated animals with lesions	11 [100]	0 0	8 [72]	7 [64]	0 0	1 [9]	11 [100] 2 [18]
Control animals with lesions	5 [62]	3 [37]	0 0	7 [87]	1 [12]	2 [25]	4 [50] 6 [75] 3 [37]
Number of lesions in vaccinated animals	69 [100]	0 0	63 [59]	43 [40]	0 0	3 [3]	96 [94] 3 [3]
Number of lesions in control animals	31 [70]	1 [29]	10 [85]	64 [1]	1 [14]	10 [20]	43 [50] 25 [29]

Table 3 cont.

E7 VACCINE

## WEEKS POST CHALLENGE

	17	21	25	29
Lesion Stage	1 2 3	1 2 3	1 2 3	1 2 3
Mean number of tumours in vaccinated group	2 [1.3] (1.1) 0.9	2.5 [1.1] (1.0) 0.7	0.3 [0.2] (1.1) 0.1	1.1 [0.5] (0.7) 0.3
Mean number of tumours in control group	1.8 [0.9] (1.1) 1.8	2 [1.1] (1.1) 1.6	0 [1.3] (1.6)	0.8 [0.4] (0.7) 0.5
Vaccinated animals with lesions	3 [27] (82) 18	5 [45] (64) 27	2 [18] (82) 18	4 [36] (72) 18
Control animals with lesions	4 [50] (62) 37	4 [50] (50) 50	0 [62] (50)	4 [37] (37) 37
Number of lesions in vaccinated animals	22 [24] (61) 14	27 [40] (43) 16	3 [6] (89) 4	12 [27] (66) 7
Number of lesions in control animals	14 [21] (33) 45	16 [26] (29) 45	0 [56] (44)	6 [30] (50) 20

a: stage 1, plaque; stage 2, papillomas &lt;2 mm; stage 3, papillomas &gt;2 mm

b: mean number of tumours in a group; SEM in parentheses

c: number of animals with lesions in a group; percentages in parentheses

d: total number of lesions in a group; percentages in parentheses

TABLE 4

E7 + L2 and L2 Vaccines  
weeks post challenge

Lesion stage	1	2	3	1	2	3	1	2	3
Animal number GROUP 1 (E7+L2)									
1	2	-	-	-	-	-	-	-	-
2	1	-	-	-	-	-	-	-	-
3	-	-	-	4	3	-	1	6	-
4-15	-	-	-	-	-	-	-	-	-
GROUP 2 (L2)									
16-26	-	-	-	-	-	-	-	-	-
27	-	-	-	1	-	-	5	1	-
28-30	-	-	-	-	-	-	-	-	-
GROUP 3 (control)									
31	5	-	-	-	6	-	5	1	-
32	1	12	-	6	6	-	-	5	8
33	5	-	-	2	8	-	3	7	-
34	6	-	-	2	7	-	2	6	4
35	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	1	-	-
37	11	-	-	-	13	-	1	6	4
38	-	-	-	-	-	-	-	-	-
39	ne			3	2	-	-	9	-
40	7	-	-	4	8	-	2	9	1
41	7	-	-	2	9	-	-	6	-
42	ne			2	5	-	1	3	4
43	ne			3	-	-	-	3	-
44	ne			-	11	-	-	10	-
45	ne			-	1	-	3	-	-
46	-	-	-	-	-	-	-	-	-
47	ne			5	7	-	1	8	-

ne = not examined; numbers represent lesions at each stage;  
lesion stages as in Table 2. -, no lesions.

CLAIMS

1. A pharmaceutical formulation for the prophylaxis of papillomavirus tumours or lesions selected from the group consisting of:

(i) a formulation which comprises a papillomavirus (PV) L2 protein or prophylactically effective fragment thereof and an aluminium compound;

10 (ii) a formulation which comprises a bovine papillomavirus (BPV) L2 protein or prophylactically effective fragment thereof and an aluminium compound;

(iii) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an aluminium compound;

15 (iv) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an adjuvant;

(v) a formulation which comprises a BPV-4 L2 protein or prophylactically effective fragment thereof and an adjuvant; and

20 (vi) a formulation which comprises a BPV-4 protein or prophylactically effective fragment thereof and an aluminium compound.

25 2. A pharmaceutical formulation according to claim 1 which comprises a PV L2 protein or a prophylactically effective fragment thereof and an aluminium compound.

3. A pharmaceutical formulation according to claim 2 wherein said PV is BPV.

5 4. A pharmaceutical formulation according to claim 3 wherein said BPV is BPV-4.

10 5. A pharmaceutical formulation according to any one of the preceding claims wherein the papillomavirus protein or prophylactically effective fragment thereof is in the form of a fusion protein with a different co-protein.

6. A pharmaceutical formulation according to claim 5 wherein the co-protein is glutathione-S-transferase (GST).

15 7. A pharmaceutical formulation according to anyone of the preceding claims wherein the papillomavirus protein or prophylactically effective fragment thereof is produced by recombinant DNA techniques.

20 8. Use of a pharmaceutical formulation according to any one of the preceding claims in veterinary or human medicine for the prophylaxis of papillomavirus tumours or lesions.

9. Use of

25 (i) a PV L2 protein or prophylactically effective fragment thereof and an aluminium compound; or

(ii) a BPV L2 protein or prophylactically effective fragment thereof and an aluminium compound; or

fragment thereof and an aluminium compound; or

(iii) a BPV-4 L2 protein or prophylactically effective fragment thereof and an aluminium compound; or

(iv) a BPV-4 protein or prophylactically effective fragment thereof and an adjuvant; or

(v) a BPV-4 L2 protein or prophylactically effective fragment thereof and an adjuvant; or

(vi) a BPV-4 protein or prophylactically effective fragment thereof and an aluminium compound

10 in the production of a vaccine for the prophylaxis of papillomavirus tumours or lesions.

10. Use of PV L2 protein or a prophylactically effective fragment thereof and an aluminium compound according to  
15 claim 9.

11. Use according to claim 10 wherein said PV is BPV

12. Use according to claim 11 wherein said BPV is BPV-4.

20

13. Use according to any one of claims 9 to 12 wherein the papillomavirus protein or prophylactically effective fragment thereof is in the form of a fusion protein with a different co-protein.

25

14. Use according to claim 13 wherein the co-protein is GST.

30

15. Use according to any one of claims 9 to 14 wherein the papillomavirus protein or prophylactically effective fragment thereof is produced by recombinant DNA techniques.

5 16. A method of immunising a mammal against papillomavirus tumours or lesions which comprises the administration of a pharmaceutical according to any one of claims 1 to 7 in a therapeutically effective dosage.

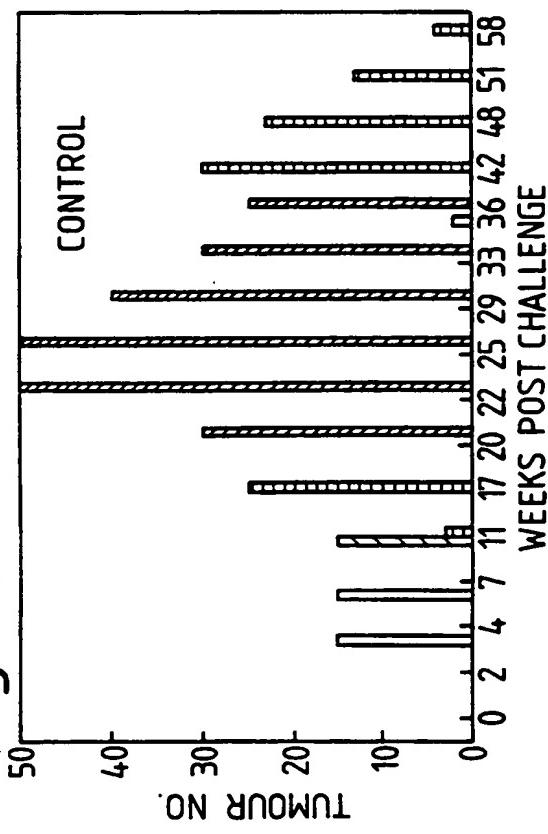
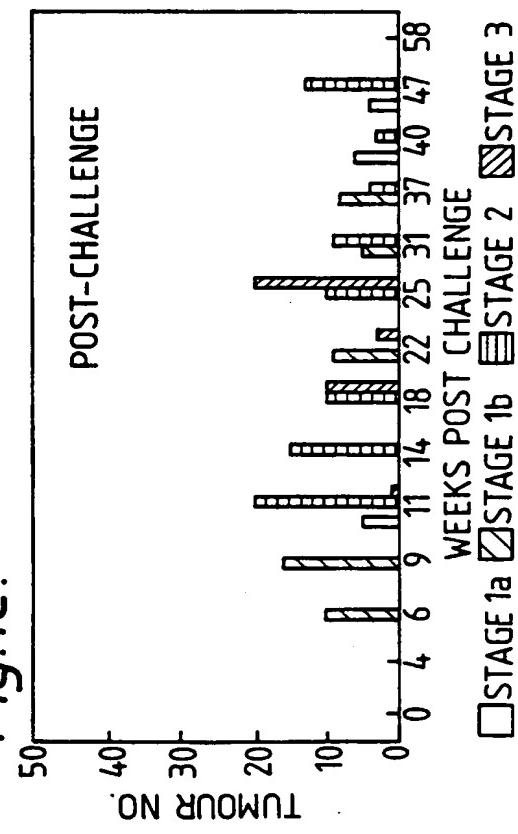
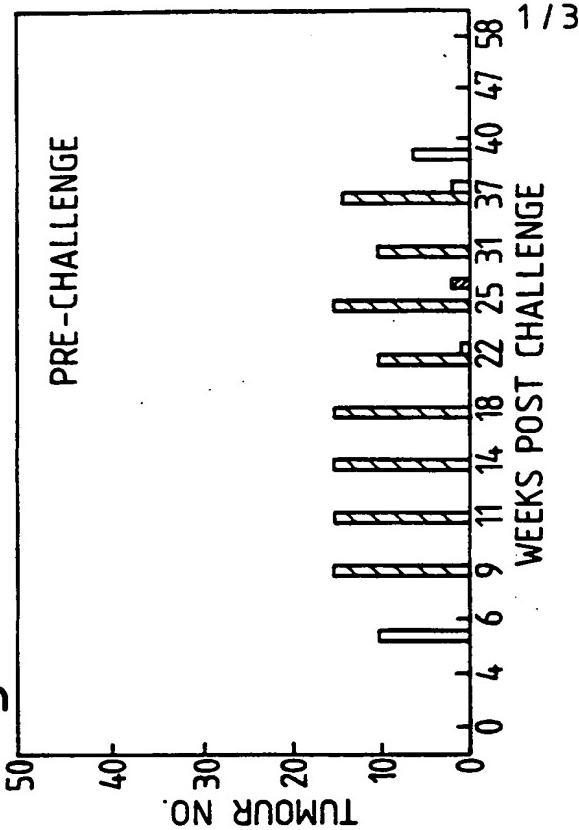
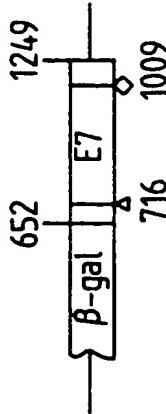
*Fig. 1A.**Fig. 1C.**Fig. 1B.**Fig. 1D.*

Fig. 2B.

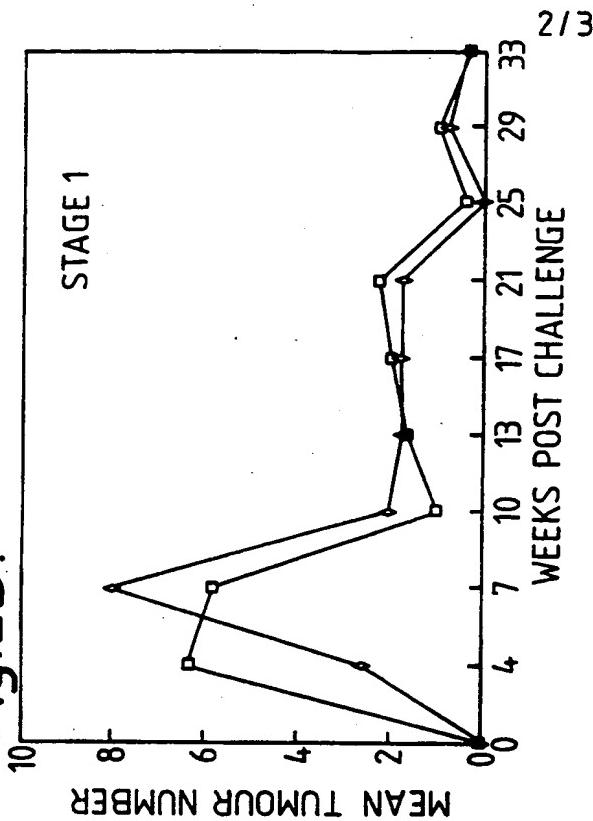


Fig. 2D.

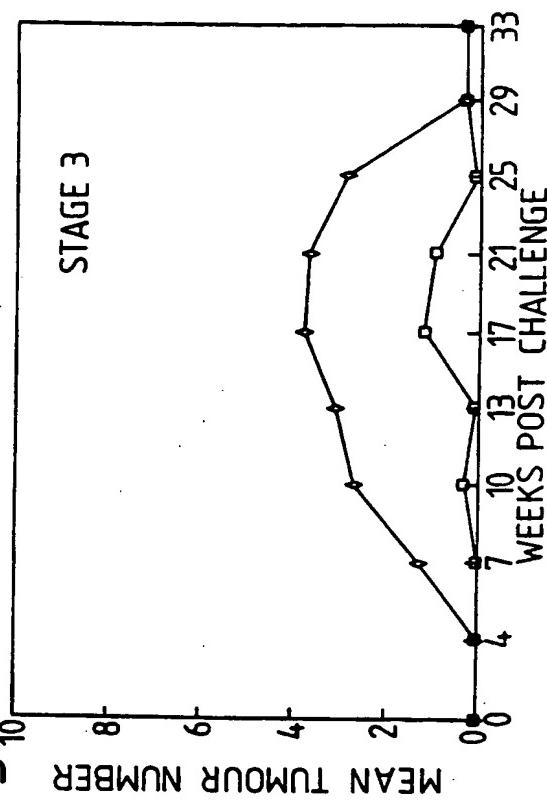


Fig. 2A.

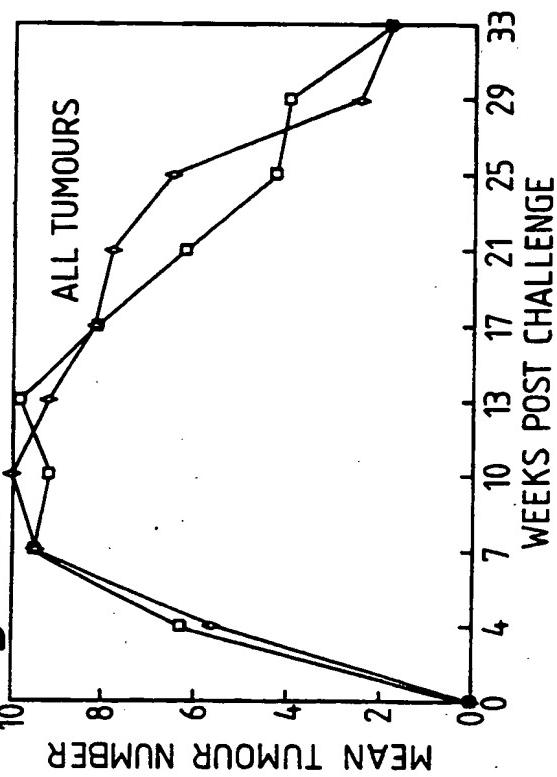
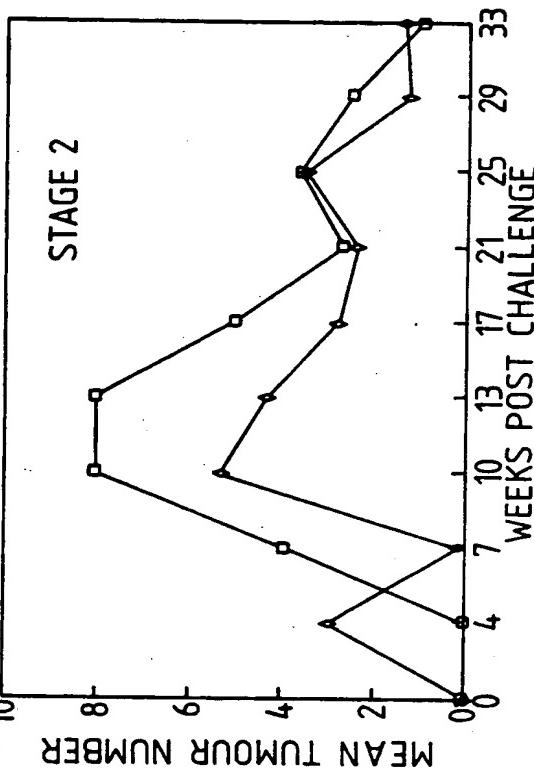
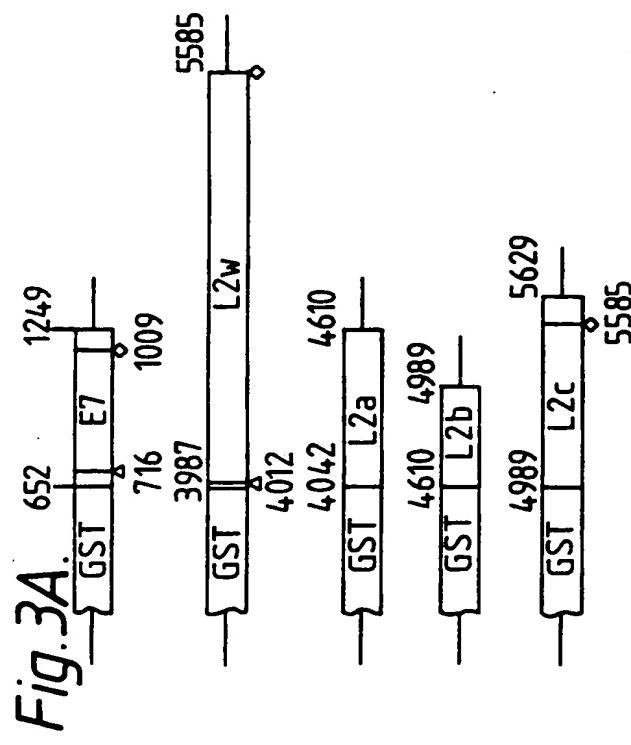


Fig. 2C.



*Fig. 3B.*

ANIMAL NUMBER	TUMOUR NUMBER
3	~6

3 / 3

*Fig. 3C.*

ANIMAL NUMBER	TUMOUR NUMBER
26	~6

*Fig. 3D.*

ANIMAL NUMBER	TUMOUR NUMBER
0	1
2	2
4	3
6	1
8	3
10	3
12	3
14	1
16	1
18	1
20	1
22	1
24	1
26	1
28	1
30	1
32	1
34	1
36	1
38	1
40	1
42	1
44	1
46	1
48	1
50	1
52	1
54	1
56	1
58	1
60	1
62	1
64	1
66	1
68	1
70	1
72	1
74	1
76	1
78	1
80	1
82	1
84	1
86	1
88	1
90	1
92	1
94	1
96	1
98	1
100	1

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 94/00683A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 C12N15/37 C12N15/62 A61K39/23 C07K13/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 00436 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 7 January 1993 see the whole document	1-16
Y	---	5,6,13, 14
X	WO,A,93 02184 (THE UNIVERSITY OF QUEENSLAND) 4 February 1993	1-4, 7-12,15, 16
Y	see page 1, line 21 - page 8, line 28	5,6,13, 14
Y	---	
X	EP,A,0 133 123 (MOLECULAR GENETICS, INC.) 13 February 1985 see page 8, line 26 - page 13, line 35 see page 16, line 17 - page 30, line 22 ---	1-16
	-/-	

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'B' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

2

Date of the actual completion of the international search  27 June 1994	Date of mailing of the international search report  14.07.94
---	--

Name and mailing address of the ISA  
European Patent Office, P.B. 5818 Patentiaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+ 31-70) 340-3016

Authorized officer

Sitch, W

## INTERNATIONAL SEARCH REPORT

Int'l	Application No
PCT/GB 94/00683	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 05248 (BRISTOL-MYERS SQUIBB COMPANY) 2 April 1992 see page 7, line 6 - page 9, line 18 see page 18, line 3 - page 19, line 13 ----	2-4, 10-12
A	EP,A,0 344 940 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 6 December 1989 see page 8, line 26 - page 9, line 54 see page 25, line 50 - page 26, line 8 ----	2-4, 10-12
A	EDITED BY CHEDID ET AL 'IMMUNOSTIMULATION,PAGES 5-22' 1980 , SPRINGER-VERLAG , BERLIN HEIDELBERG NEW YORK see page 13, paragraph 2 - page 14, paragraph 1 ----	2-4, 10-12
A	EP,A,0 375 555 (MEDGENIX GROUP,S.A.) 27 June 1990 see the whole document ----	
P,X	WO,A,93 20844 (CANCER RESEARCH CAMPAIGN TECHNOLOGY LTD.) 28 October 1993 see the whole document -----	1-16

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/GB94/00683

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark :** Although claim 16 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Appl. No.

PCT/GB 94/00683

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300436	07-01-93	AU-A- EP-A-	1985992 0592480	25-01-93 20-04-94
WO-A-9302184	04-02-93	EP-A-	0595935	11-05-94
EP-A-0133123	13-02-85	NONE		
WO-A-9205248	02-04-92	AU-A- CN-A-	8762991 1067382	15-04-92 30-12-92
EP-A-0344940	06-12-89	US-A- AU-B- AU-A- JP-A-	5180806 628337 3481389 2096593	19-01-93 17-09-92 16-11-89 09-04-90
EP-A-0375555	27-06-90	FR-A- CA-A- JP-A-	2641081 2006118 2291297	29-06-90 23-06-90 03-12-90
WO-A-9320844	28-10-93	AU-B-	3897093	18-11-93

**THIS PAGE BLANK (USPTO)**